Heterogeneity of Human Whole Blood Platelet Subpopulations. I. Relationship Between Buoyant Density, Cell Volume, and Ultrastructure

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A quantitative high yield method utilizing isosmotic arabino-galactan (Stractan) solutions and isopycnic centrifugation was developed to isolate and to fractionate total human platelet populations into density-dependent subpopulations. Isolated platelets were free of factor VIII/von Willebrand factor and other plasma proteins. They responded to ADP, epinephrine, and collagen with a sensitivity equal to platelet-rich plasma platelets. The correlation of platelet density with volume and ultrastructure was examined for normal subjects. Recovery of total platelet populations averaged 92.76% ± 3.64% (SD). Normal individuals exhibited a narrow range of platelet buoyant density distribution. Computerized probability plot analysis of platelet volume distribution for 15 normal subjects' total platelet populations showed a mean volume of 5.17 ± 0.46 cu μm (SD). Platelets with buoyant density ≤ 1.062 g/ml had a mean volume of 4.50 ± 0.48 cu μm, while platelets with buoyant density > 1.071 g/ml, but ≤ 1.084 g/ml, had a mean volume of 5.32 ± 0.63 cu μm (SD). The volume difference by paired t test was significant, p > 0.001. Thin-section electron microscopy demonstrated a significant reduction of granule content in light platelets, as compared to heavy platelets, but an equal number of mitochondria for both groups. These differences of platelet volume and structure between light and heavy platelets suggested that aging may be a determinant of platelet heterogeneity.

EXPERIMENTAL DATA accumulated over the past decade support the observation that mammalian platelets are heterogeneous. There is heterogeneity of cell volume, buoyant density, metabolism, protein synthesis, lipid peroxidation, and hemostatic function. Kinetic studies in man and animals suggest that platelets have a finite life span and age in vivo by a process that is independent of random destruction. Age-specific cohort radionuclide label studies support the hypothesis that platelet heterogeneity is the result of in vivo platelet aging; however, the biologic regulators of platelet senescence remain obscure.

Experimental models of platelet aging have relied upon alteration of steady-state thrombopoiesis to produce populations of young and old platelets. As a result of megakaryocyte alteration during stress, platelets formed under these conditions may be biologically different from unstressed platelets. This artifact has been avoided by exploiting the relationship between platelet age and buoyant density in order to derive age-specific platelet cohorts without alteration of thrombopoietic equilibrium. Unfortunately, existing techniques...
have not permitted optimal utilization of platelet age-dependent buoyant density separation. Platelet-rich plasma (PRP) preparation specifically excludes large, dense platelets which results in the selection of a biased sample for initiation of subsequent investigations. Isolation of age-specific platelet cohorts requires quantitative, efficient recovery of a total whole blood platelet population by a means that preserves cell function and structure.

The purpose of the present experiment was to develop a method for high-yield quantitative isolation of total platelet populations from whole blood. Previous studies using the polysaccharide arabino-galactan (Stractan) had shown that red cells could be separated into age-specific cohorts by buoyant density centrifugation. Based upon the analogous relationship between platelet age and density, this methodology was adapted to platelet isolation. Subsequently, we investigated the buoyant density profile of total platelet population isolates from a group of normal individuals and examined the relationship of platelet buoyant density with cell volume and ultrastructural morphology in order to evaluate the importance of platelet aging as a cause of platelet heterogeneity.

MATERIALS AND METHODS

Sample Acquisition

Platelets were isolated from the whole blood of 15 normal individuals, eight females and seven males, ranging in age from 20 to 53 yr. All subjects had normal complete blood counts (including platelet counts), were free of known disease, and had abstained from all medications for 10 days prior to the study. Samples were obtained from female subjects without respect to menstrual cycle chronology. Subjects were bled less than 50 ml during the 30 days prior to sampling for platelet isolation. Whole blood was obtained by venipuncture with an 18-gauge needle and plastic syringe (Sherwood Medical Industries, Deland, Fla.). The blood was immediately transferred to plastic tubes (No. 2074, Falcon, Oxnard, Calif.) and anticoagulated with 4% sodium citrate dihydrate (pH 6.76), and 1:90 diluted blood. All procedures were carried out at room temperature.

Preparation of Stractan Solutions

Crude Stractan (St. Regis Paper, Tacoma, Wash.) was purified as previously described. One volume of 0.3 M K$_2$HPO$_4$-Na$_2$HPO$_4$, pH 7.4 buffer, was added to 19 volumes of deionized Stractan solution, and the pH of the Stractan solution was adjusted to 7.4 ± 0.05 with 1 N NaOH. The Stractan solution was diluted to a 30% concentration with deionized water. Two hundred milligrams of glucose, 400 mg of sodium citrate dihydrate, and 3 g of bovine serum albumin (fraction V, Pentex, Kankakee, Ill.) were added per 100 ml available water. The pH was readjusted to 7.4 ± 0.05 with 1 N NaOH.

The osmolarity of the 30% Stractan solution was measured with an osmometer by freezing point depression as previously described. The osmotic deficit was calculated, and the amount of desiccated NaCl required to adjust the 30% Stractan solution to isosmolarity (290 ± 1 mOsm/liter) added. The final 30% Stractan solution was filtered through an 0.2-μm filter to remove bacteria and was stored in 50-ml aliquots at -20°C. Working solutions of 20%, 17%, 16%, 15%, and 10%, with respective specific gravities at 22°C of 1.084, 1.071, 1.066, 1.062, and 1.042 g/ml were prepared by dilution of 30% (1.127 g/ml) Stractan with an isosmotic buffered saline glucose citrate solution (BSG-citrate) (NaCl 6.832 g/liter, sodium citrate dihydrate 4.0 g/liter, glucose 2.0 g/liter, Na$_2$HPO$_4$ 1.22 g/liter, K$_3$PO$_4$ 0.218 g/liter, pH 7.40 ± 0.05, osmolarity 290 ± 1 mOsm/liter). A density calibration curve was prepared using these solutions. The working solutions were divided into 50-ml sterile screw-cap tubes (No. 2074, Falcon, Oxnard, Calif.) and stored at -20°C. Sterile BSG-citrate solution was also stored at -20°C.
Platelet Separation

Total platelet separation from whole blood was performed in a two-step process. Ten milliliters of whole blood containing 0.4% sodium citrate was centrifuged at 600 g for 3 min (20°C) to prepare PRP. The PRP was removed with a plastic pipette (Falcon, Oxnard, Calif.), and the red cell pellet, diluted with 10% Stractan solution to original volume, was layered over an equal volume of 20% Stractan solution in a ⅛ in. × 4 in. cellulose nitrate tube (Beckman, Palo Alto, Calif.). This single-step discontinuous gradient was centrifuged at 10,000 rpm for 10 min at 20°C in an SW 27.1 Rotor (Beckman Instruments, Palo Alto, Calif.). Platelets sedimented at the 1.084 g/ml interface. The tube was sliced (Tube Slicer Assembly, Nuclear Supply and Service, Washington, D.C.) above the plasma-platelet boundary and below the platelet-Stractan boundary (Fig. 1A). The cell layers were washed out with small amounts of BSG-citrate solution and pooled with the PRP fraction to yield a total platelet population.

When rigorous separation from plasma proteins was required, the total platelet population was diluted with BSG-citrate and layered over a discontinuous gradient consisting of 4 ml 20% Stractan and 3 ml 10% Stractan. The tube was centrifuged at 10,000 rpm at 20°C for 10 min in the Beckman SW 27.1 Rotor. Plasma proteins remained at the plasma 1.042 g/ml layer while platelets traversed the 1.042 g/ml Stractan layer and were isolated as before by removal of the 1.084 g/ml interface.

The total platelet population was subfractionated into four populations on a discontinuous Stractan gradient of 20% (1.084 g/ml), 17% (1.071 g/ml), 16% (1.066 g/ml), and 15% (1.062 g/ml), respectively, and centrifuged for 30 min at 20°C at 20,000 rpm in the SW 27.1 rotor. The rotor was allowed to coast to a stop, the tubes were withdrawn, and the four bands were sliced out (Fig. 1B).

Platelet Counts

Platelet counts were done in duplicate by visual phase contrast and electronic methods. Visual platelet counts were performed under phase-contrast microscopy (Bausch and Lomb, Rochester, N.Y.) with improved Neubauer chambers (American Optical, Buffalo, N.Y.) using 1% ammonium oxalate. Electronic platelet counts were performed for all samples except whole blood and red cell pellet fractions by a modification of the method of Bull et al. Owing to equipment changes in the laboratory, the following machines were used: Coulter B (Coulter Electronics, Hialeah, Fla.) with 30-μm orifice, aperture current = ½, amplification = ½, matching switch 64L, gain = 100, lower threshold = 5, upper threshold = 100; Coulter ZBI with 30-μm orifice, aperture current = 1, amplification = 2, matching switch = 1:40, gain = 80, same thresholds as for Coulter B; Cellozone cell counter (Particle Data, Elmhurst, Ill.) 30-μm orifice tube with aperture current = ¼, amplification = 68, logarithmic scale at four doublings, lower threshold = 6, upper threshold = 100. Machines were standardized daily using 2.02-μm-diameter latex spheres (Coulter Electronics, Hialeah, Fla.) and platelet reference standards 300,000 ± 20,000/μl.
and 70,000 ± 20,000/μl (General Science, Bridgeport, Conn.). Electronic platelet counts were performed in triplicate. Dilutions were made with Isoton counting solution (Coulter, Hialeah, Fla.) which was filtered through a 0.2-μm grid (Nalge, Sybron, Rochester, N.Y.). Counts were adjusted for Coulter machines ≤ 10,000/100 μl sample and for the Particle Data instrument ≤ 14,000/50 μl, so that coincidence error was ≤ 1%. Final counts were averaged, corrected for background, and averaged with phase-contrast counts. Visual and electronic counts agreed within 5%.

Platelet Volume Determination

Measurement of platelet volumes and volume-frequency distributions was carried out by two methods. Initial studies were performed with a Coulter Channelyzer equipped with ZBI counter (Coulter Electronics, Hialeah, Fla.). This machine was calibrated with 2.02-μm-diameter latex spheres. The accuracy of stated latex sphere size was determined by electron microscopy. By manipulation of amplification, it was possible to obtain two calibration points at the extreme ends of the scale and to demonstrate that the scale was linear from 1.8 to 18 cu μm. All sizing was performed at 23°C with a 30-μm-diameter orifice, and samples were diluted with filtered Isoton to give total particle counts of 7500 per 100-μl sample. Data were accumulated to a peak of 4000 particles in 100 windows. Frequency distribution curves were plotted by use of a linear plotter, and the curves were divided into five channel segments for integration. Cumulative frequency distributions were plotted against midpoint logarithmic volumes for each segment and converted to a probability plot analysis.27 Mean volumes were derived from these plots.

Later studies were performed with a Particle Data Cellozone computerized system with logarithmic amplifier, which greatly improved the resolution of platelet size and produced a linear size range over ten volume doublings. The Particle Data machine provided computerized programs for obtaining frequency distributions, mean, mode, median, cumulative frequency distribution, and probability plot analysis. All sizing was performed at 23°C with a 30-μm orifice. Platelets were diluted with filtered Isoton solution, and total particle counts were adjusted to less than 14,000/50-μl sample to maintain a coincidence error of less than 1%. Data were accumulated in 384 channels to a peak count of 2000 particles.

Demonstration of Platelet Isolation From Plasma Proteins

Total platelet populations were isolated free of plasma proteins as described above. Two methods were utilized to demonstrate freedom from plasma proteins: (1) separation of platelets from 125I-labeled human fibrinogen, and (2) absence of platelet aggregation in response to the antibiotic ristocetin (Abbott Laboratories, North Chicago, Ill.).

Labeled fibrinogen. Normal human fibrinogen, prepared by ammonium sulfate fractionation,28 was labeled with 125I by the lactoperoxidase method.29 The final fibrinogen was 86% coagulable. One-half milliliter of 125I-fibrinogen (2.0 μCi, 33 mg/ml protein) was mixed with 10 ml of citrated whole blood and incubated at room temperature for 30 min, platelets were isolated free of plasma proteins as described, and the supernatant fraction and the four platelet fractions were counted for radioactivity (Nuclear-Chicago, Des Plaines, Ill.).

Ristocetin response. Platelets isolated from plasma proteins as described were adjusted to a final count of 200,000/μl with BSG-citrate solution. Aggregation was measured in an aggregometer (Chronolog, Broomall, Pa.) at 37°C using Teflon-coated stir bars at 1000 rpm. Aliquots of platelet suspension (0.45 ml) were tested with ristocetin at a final concentration of 1.5 mg/ml. Exogenous purified Factor VIII/von Willebrand factor (VWF)30 was added to a final concentration of 15 μg/ml to demonstrate ristocetin response restoration.31

Preparation of Platelets for Aggregation Studies

A total platelet population was isolated as previously described. The fraction was diluted with BSG-citrate to reduce the Stractan concentration below 10% and layered over a two-stage discontinuous gradient of 20% and 10% Stractan. This gradient was centrifuged in the SW 27.1 rotor for 10 min at 10,000 rpm to provide a platelet concentrate free of plasma proteins, granulocytes, and red cells. The total platelet population free of plasma proteins was diluted with 6 volumes calcium-free Tyrode's buffer (pH 7.4, 270 mOsm/liter) and layered over 2.5 ml of 35% albumin
HUMAN PLATELET SUBPOPULATIONS (pH 7.4, 290 mOsm/liter). The albumin cushion was prepared by dissolving 35 g of Pentex fraction V bovine serum albumin in 65 g of deionized water. The pH was adjusted to 7.4 with 1N NaOH, and the osmolarity was adjusted to 290 mOsm/liter by the addition of desiccated NaCl. The platelets were pelleted onto the albumin cushion by centrifugation at 600 g for 15 min at 20ºC. The platelet-poor supernatant fraction was recovered and processed once again to maximize platelet recovery. The platelets were aspirated from the albumin cushion and resuspended in autologous platelet-poor donor plasma. Final platelet count was adjusted to 300,000/µl. PRP was prepared at 600 g for 3 min at 20ºC to serve as the control.

Platelet response to ADP, epinephrine (Sigma Chemical, St. Louis, Mo.), and collagen (Worthington Biochemical, Freehold, N.J.) was measured with a Payton Aggregometer (Payton Instruments, Buffalo, N.Y.) by the method of Born and Cross,32 using the Teflon-coated stir bars at 1000 rpm and 37ºC. The final pH of the platelet suspension was adjusted to 7.6 with 0.1N NaOH prior to use. Responsiveness was determined at multiple concentrations of ADP and epinephrine in the range from 0.1 μM to 10 μM and for collagen from 15.6 μg/ml to 156 μg/ml. Stractan and PRP platelet preparations were assayed in parallel, and the minimal concentrations of aggregating agents that produced comparable maximal aggregation were determined.

Preparation for Electron Microscopy

Specimen groups consisted of the four platelet subpopulations obtained by Stractan separation. Each subpopulation, which contained at least 130,000 to 200,000 platelets/µl, was diluted 1:10 with 3% glutaraldehyde in 0.066 M phosphate buffer, pH 7.4, and was fixed for 45 min at 4ºC. After concentration by centrifugation (1465 g for 7 min), the platelets were washed overnight in phosphate buffer at pH 7.4 and postfixed the following day in 1% osmium tetroxide for 1 hr at 4ºC. Specimens were dehydrated by washing in increasing concentrations of ethanol and propylene oxide. After 24-hr infiltration with a 50% Epon-50% propylene oxide solution, the platelets were embedded in Epon and cured for 48 hr. All Epon material was sectioned on a LKB Ultramicrotome (LKB 4800A, Ultratome, LKB Produkter, Stockholm) to silver-gold interference thickness. Sections were placed on Formvar-coated copper grids and observed with a Zeiss 10 electron microscope (Carl Zeiss, New York, N.Y.).

Technique of Quantitative Granule Count of Platelet Electron Micrographs

Electron micrographs were obtained of continuous, random fields of platelets at a negative magnification of 4000 x. In addition, selected micrographs of small numbers of representative platelets were obtained at negative magnifications of 10,400 x and 25,600 x. At least 110 platelets per subpopulation for each normal subject were evaluated and photographed. It was assumed that examining a large number of cells would randomize the variation in level of section through platelets and would yield an unbiased estimate of organelle content. Granules were counted by direct observation or by examination of negatives, using a wide-field dissecting microscope at low magnification. Recognizable dense bodies were included in the granule count. Mitochondria were counted as separate organelles. The mean number of organelles per platelet for each subpopulation was calculated, and differences among populations were statistically analyzed by the paired t test.33

Control Platelets Obtained for Comparison with Stractan Separation

Ten-milliliter specimens were obtained by directly dripping blood from the venipuncture needle into 100 ml of 3% glutaraldehyde in 0.066 M phosphate buffer, pH 7.4. Specimens were immediately centrifuged at 525 g for 10 min to remove erythrocytes, and PRP was transferred to fresh 3% glutaraldehyde in 0.066 M phosphate buffer, pH 7.4. After 45 min fixation at 4ºC, specimens were centrifuged at 2520 g to concentrate platelets. The remaining procedure for electron microscopy was identical to that described above.

RESULTS

Comparative Yields and Volumes of PRP and Stractan Platelets

PRP was prepared by a standard technique (600 g for 3 min at 20ºC) from the citrated whole blood of ten normal subjects to measure the variation in
platelet yields for this method. The observed range of platelet loss was 17.15% - 35.75% of the total available platelets. The average platelet loss was 25.77% ± 5.34% (SD).

Platelet isolation from 15 normal subjects using a simple two-step Stractan discontinuous gradient of limit density 1.084 g/ml permitted a 92.6% ± 3.64% (SD) recovery of the total available platelets from 20 ml whole blood. The observed range of recovery was 90.40% - 97.81%. Previous experiments limited to single tube procedures gave recoveries as high as 97%. Modification of the earlier technique maximized platelet retention by the 1.084 g/ml interface, such that the residual red cell pellets contained no platelets under phase microscopy. Lymphocytes have a buoyant density distribution identical to that of platelets, while granulocytes sediment with the red cells. Recovery and distribution of leukocytes provided an independent means to assess the efficiency of the isolation procedure. Total leukocyte recoveries averaged 94.70% ± 2.60% for 15 normal subjects.

Prior to measurement of platelet volumes, it was necessary to ascertain if exposure of platelets to arabino-galactan caused changes in platelet volume. Equal volumes of PRP (centrifuged at 600 g for 3 min, 20°C) were incubated for 150 min with equal volumes of either platelet-poor plasma or 20% Stractan solution at laboratory temperature. Platelet volume was determined at zero time and after 150 min of exposure to the respective diluent. Platelet mean volume and volume distribution did not change significantly over time after Stractan exposure.

A comparison of platelet volumes for PRP platelets and for the platelets excluded during the preparation of PRP, but recovered subsequently from the red cell pellet at the 1.084 g/ml interface demonstrated the diminished mean, modal, and overall volume distribution of PRP platelets, as compared to the Stractan platelets (Fig. 2). This decrement of platelet mean volume was augmented as centrifugal force was increased, as reflected in decreased mean volumes at a variety of centrifugal forces routinely used to prepare PRP. The mean volume of PRP platelets prepared at 540 g/min was 6.63 cu μm, but decreased to 4.13 cu μm when platelets were prepared at 4800 g/min. Although this artifact could be partially circumvented by preparing PRP at low centrifugal forces, the yield of platelets was greatly reduced.

Platelets isolated by the Stractan method were free of plasma proteins. Recovery of 125I-fibrinogen from the gradient was 97.2% of the starting material.
Table 1. Platelet Subpopulation Buoyant Density Distribution for 15 Normal Subjects

<table>
<thead>
<tr>
<th>Density (g/ml)</th>
<th>Per Cent Total Available Platelets</th>
</tr>
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<tbody>
<tr>
<td>I, ≤ 1.062</td>
<td>17.42 ± 6.68*</td>
</tr>
<tr>
<td>II, ≤ 1.066</td>
<td>38.61 ± 7.05</td>
</tr>
<tr>
<td>III, ≤ 1.071</td>
<td>29.95 ± 6.67</td>
</tr>
<tr>
<td>IV, ≤ 1.084</td>
<td>14.03 ± 3.58</td>
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</table>

Distribution and recoveries are calculated based on total platelet population [average recovery, 92.14% ± 4.91% (SD)].

No radioactivity was associated with the platelet subfractions. Platelets separated by this method did not aggregate in response to the addition of the antibiotic ristocetin, nor did they aggregate in response to purified factor VIII/VWF in the absence of ristocetin. When both purified Factor VIII/VWF and ristocetin were added to the platelet suspension, a full response occurred. Autologous donor plasma was capable of restoring the ristocetin response.

Density Distribution

Total whole blood platelet populations from 15 normal subjects were fractionated into 4 subpopulations on discontinuous gradients at densities 1.062 g/ml, 1.066 g/ml, 1.071 g/ml, and 1.084 g/ml. These densities were arbitrarily chosen to provide two extreme populations of approximately 15% each, and two middle populations of approximately 30% each of the total available platelets. Platelets from these 15 individuals were distributed as shown (Table 1). Total platelet recovery for this separation was 92.14% ± 4.91% (SD). Hematologically normal individuals exhibited a well-defined range of platelet buoyant densities. Repeated separations of the platelets from the same individual gave reproducible results for males. Menstruating females, however,
Table 2. Platelet Population Mean Volume as Determined by Probit Analysis

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean Volume (cu μm)</th>
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<tbody>
<tr>
<td>Total</td>
<td>5.17 ± 0.46*</td>
</tr>
<tr>
<td>I</td>
<td>4.50 ± 0.48</td>
</tr>
<tr>
<td>II</td>
<td>5.19 ± 0.51</td>
</tr>
<tr>
<td>III</td>
<td>5.48 ± 0.54</td>
</tr>
<tr>
<td>IV</td>
<td>5.32 ± 0.63</td>
</tr>
</tbody>
</table>

Platelet size was determined by probit plot analysis of cumulative frequency distributions versus midpoint log volumes using the Coulter Channellyzer system. This system was calibrated with latex spheres; diameter = 2.02 μm.

*SD.
†Groms per milliliter.

demonstrated minor variation in density distribution in relation to the chronology of their menstrual cycle.

Relation Between Platelet Buoyant Density and Volume

The distribution of platelet volume for a single individual’s total platelet population (Fig. 3) exhibited a wide range (1.44-44.65 cu μm). The range of total platelet population mean volumes for the 15 normal subjects as determined by probability plot analysis of the Channellyzer data was 4.08-5.84 cu μm, with an average mean volume of 5.17 ± 0.46 cu μm (SD) for the group. Subsequent determination of total platelet population mean volume using the logarithmic amplifier for 34 normal subjects showed a range of 5.42 to 8.17 cu μm, with an average mean volume of 6.53 ± 0.72 cu μm (SD).

The mean volumes of the four platelet subpopulations separated by density is shown in Table 2. There was considerable overlap of subpopulation volumes among the group of subjects studied, but each subject showed a general trend of increasing volume with increasing density. The degree of volume overlap between the lightest and heaviest platelets for a single subject was clearly demonstrated (Fig. 4). Statistical analysis by paired t test showed that the lightest platelets were significantly smaller than the three heavier populations, with the largest difference between the first and third layers (Table 3). Improved volume

![Fig. 4. Comparison of the volume distribution of the lightest (e) and the heaviest (o) platelets from a single subject isolated in a Stractan citrate system. Volume was determined at 23°C with a logarithmic amplifier calibrated as before. Red cell and lymphocyte contamination was less than 0.5%. Ordinate indicates frequency; abscissa indicates volume in cu μm.](image)

*Corash L: Unpublished observation.
Table 3. Significance of Subpopulation Mean Logarithmic Volume Differences by Paired t Test for 15 Individuals

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Mean Log Volume Difference</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>0.0</td>
</tr>
<tr>
<td>II</td>
<td>+7.3796*</td>
</tr>
<tr>
<td>III</td>
<td>+9.4610*</td>
</tr>
<tr>
<td>IV</td>
<td>+6.6221*</td>
</tr>
</tbody>
</table>

Statistical analysis of comparison of subpopulation platelet volumes by the paired t test. The significance level is indicated. The direction of the sign indicates "smaller than" for negative and "larger than" for positive signs. The actual magnitude of the t value is given for comparison of differences.

* p ≤ 0.001.
† 0.05 > p > 0.025.
§ Not significant.

Platelet Aggregation

Platelet aggregation in response to collagen, ADP, and epinephrine was measured (Fig. 5). Stractan-isolated platelets were equivalent to untreated PRP control platelets when aggregated by the three agents tested. The minimal agent dose that produced maximum response for the parallel control in one subject is shown. Lower concentrations of ADP produced a well-defined double wave, but the total maximal response was less than that shown in Fig. 5B.

Platelet Ultrastructure

Platelets not exposed to Stractan showed oval or discoid shapes with smooth, nonprojecting plasma membranes. Granules were scattered throughout the cell interior between glycogen particles, microtubules, and the surface canalicular system (SCS). Stractan-treated platelets demonstrated varying amounts of organelles and internal structures, depending on the subpopulation examined (Figs. 6A–D). Unfractionated Stractan-isolated platelets appeared similar in all respects to the control platelets, except that the former usually had at least one thin cytoplasmic pseudopod like projection.

Stractan-isolated platelet subpopulations differed in the prominence of SCS, number of granules, number of dense bodies, and concentration of glycogen particles. The SCS was most prominent in the least dense platelet subpopulation (Fig. 6A). Many platelets from this fraction demonstrated at least 50% of the cross-sectional area to consist of the SCS. In progressively denser subpopulations, the SCS occupied a proportionately smaller estimated fraction of the total cross-sectional area. In the densest platelet subpopulation, the SCS occupied approximately 10% of the total area (Fig. 6D). Platelet granules appeared more numerous in the densest subpopulation. Granules were scattered throughout the platelet cytoplasm in all subpopulations. No morphological evidence of aggregation was noted. No qualitative differences were noted between granules for different subpopulations.
Mitochondria and total granules (lysosomal granules and dense bodies) were quantitatively measured for each platelet subpopulation (Table 4). There was a constant number of mitochondria per platelet regardless of buoyant density, but granule content increased with increasing density. Even the least dense platelets were morphologically intact, with demonstrable cellular organelles. Platelet fragments were not evident. Statistical analysis of platelet subpopulation organelle content showed that the differences between subpopulations for
Fig. 6. Platelet ultrastructure subpopulations, designated I, II, III, and IV, ranging from least to most dense, respectively. All platelets show minimal to moderate pseudopod formation. (A) Platelets from population I. Note surface canalicular system (V), occasional mitochondria (M), and infrequent granules. A relatively large amount of the platelet cross-sectional area is occupied by surface canalicular system, and there is a lack of large clusters of glycogen particles. × 9000. (B) Platelets from population II. Note somewhat decreased amounts of surface canalicular system (V) and increased numbers of granules (g) in platelets. × 12,000. (C) Platelets from population III demonstrating increased numbers of platelet granules as well as focal collections of glycogen particles. Note relatively smaller size of surface canalicular system as compared with population I cells. × 12,000. (D) Platelets from population IV showing mitochondria (M), granules (g), and glycogen particles filling cell cytoplasm. Note close approximation of granules (upper right inset) as well as microtubular system (lower right inset). × 9000; insets × 12,000.
Table 4. Ultrastructural Analysis of the Four Platelet Subpopulations: Relationship of Granule and Mitochondria Content With Buoyant Density

<table>
<thead>
<tr>
<th>Platelet Subpopulation</th>
<th>n*</th>
<th>Granules</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I*</td>
<td>1140</td>
<td>2.03 ± 0.54</td>
<td>0.76 ± 0.45</td>
</tr>
<tr>
<td>II*</td>
<td>1724</td>
<td>3.27 ± 0.82</td>
<td>0.57 ± 0.28</td>
</tr>
<tr>
<td>III*</td>
<td>1604</td>
<td>4.82 ± 1.11</td>
<td>0.57 ± 0.16</td>
</tr>
<tr>
<td>IV*</td>
<td>1669</td>
<td>6.48 ± 1.36</td>
<td>0.66 ± 0.21</td>
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</tbody>
</table>

*n equals total number of platelets evaluated per subpopulation.
†Granules and mitochondria are expressed as the mean number per platelet for each subpopulation based upon total granule or mitochondria number per total number of platelets counted; SD is given for each mean. Nine subjects were studied.

Granule content were highly significant (0.005 > p > 0.001 and p < 0.001), while there was no significant statistical difference for mitochondria distribution among the subpopulations.

DISCUSSION

Inert oils,3'34'35 sucrose,3638 and albumin20 have been the principal materials used in earlier platelet isolation techniques. One cannot quantitatively compare the Stractan system with those density separation methods that use PRP as the source of a total platelet population. However, some qualitative comparisons are possible. Silicone oils34,35 isolate platelets from whole blood, but at a lower density than Stractan. This difference of separation densities may reflect an alteration of cell volume by the oil. In addition, the inert oils are difficult to remove from platelets. The Apiezon-A-n-dibutyl phthalate system3 causes fewer problems, but has only been used with PRP. The albumin method is suitable for producing density-dependent platelet subpopulations from PRP, but has not been used to isolate whole blood platelets. Isosmolar albumin solutions are more expensive and are considerably more difficult to prepare than Stractan solutions.

The sucrose systems,3638 which are hyperosmolar, do not isolate platelets from whole blood. Sucrose changes platelet buoyant density due to loss of cell water and adversely affects ultrastructural morphology.38 Recent work by Boneu et al.37 with a sucrose-PRP technique has failed to confirm a relationship between platelet age and buoyant density. However, in view of the artifacts caused by PRP and sucrose, this conclusion may not be valid. Observations from other laboratories19,20 performed under isosmolar conditions strongly support the relationship between platelet age and density. The Stractan whole blood technique provides an improved means to isolate density-dependent platelet cohorts.

The deficiencies of existing systems for the removal of plasma proteins from platelets have been described.39 These deficiencies are avoided by the Stractan technique, which isolates a total platelet population and frees it of the polysaccharide by concentration onto an isosmolar albumin cushion (290 mOsm/liter, pH 7.4). The method described by Walsh40 appears to separate platelets from plasma proteins. Unlike the Stractan method, this technique uses PRP and an albumin cushion of unspecified osmolarity. The latter difference may account for the ultrastructural changes and variable aggregation response re-
ported by other workers with that system. Thé Ganguly and Sonnichsen method yields responsive platelets from PRP, but it uses hyperosmolar sodium metrizoate, which must then be removed by a 2-hr dialysis.

Stractan-isolated platelets were free of plasma proteins, including macromolecules as opposed to the gel filtration method in which Factor VIII/VWF co-chromatographed with the platelet fraction. Addition of purified Factor VIII/VWF to Stractan-separated platelets fully restored the ristocetin response. Resuspension of the platelets in autologous or homologous plasma restored sensitivity to epinephrine, ADP, and collagen-mediated aggregation, which was comparable to that of control PRP.

There are numerous reports of the measurement of platelet volumes. A variety of methods have been used to date: electronic sizing, thrombocrit determination, and direct microscopic measurement. We feel that electronic sizing provides the most complete estimate of particle size for small amounts of cells. With appropriate orifice size, amplifier rise time, and transit time, the measurement of cell volume by electric zone sensing instruments is accurate. The thrombocrit method of Born and similar techniques provide a mean cell volume but are subject to the artifacts of particle fragmentation and plasma trapping. While the latter is correctable by isotopic studies, the other effect is not measurable; the technique requires a concentrated particle suspension often not obtainable and yields only a mean volume without any information as to size distribution.

Our initial platelet volume data acquired with the linear amplified Coulter Channelizer system required probability plot transformation for analysis, and was not as accurate as the logarithmic Particle Data apparatus owing to poor definition in the smaller platelet range and to exclusion of the largest platelets because of the limited number of volume doublings in the linear mode. This limitation has also been reported by other workers. The logarithmic system (range 0.27–260.48 cu μm) allowed definition of the smallest platelets while including the largest platelets. These technical differences accounted for the minor difference in mean volumes between the probability plot-derived data (5.17 ± 0.46 cu μm) and the direct logarithmic-derived data (6.53 ± 0.72 cu μm). We agree with the observations of other workers that calibration of the particle analyzer with red cells as opposed to latex spheres gives larger volumes. The ratio between the two standards for our system was 0.58; however, we have chosen to use latex spheres because of greater reproducibility and ease of performance.

The present experiments report for the first time measurements of platelet volumes from total platelet populations. Data from several laboratories have suggested that platelet volume in the unstressed state is distributed in a log normal manner. Our estimates of platelet mean volume, using a logarithmic system, agree with those of Paulus, who has measured platelet volumes derived from low speed PRP (40 g for 25 sec). We also have found good agreement between total isolated platelet mean volumes and the mean volume derived from blood allowed to sediment at unit gravity in a capillary tube until a 3 μl sample of red cell–free plasma is obtained. We have found a larger range of platelet volume than that described by other investigators, owing to the ability to isolate
a total platelet population and the improved resolution of the logarithmic system. We must conclude that these volume measurements are relative and not absolute; even when the system is calibrated with a biologic membrane (red cells), there may still be membrane differences for platelets, which can affect absolute volume determination.

Data derived from the Stractan system show that lighter platelets are significantly smaller than heavier platelets for all positions in the gradient except the third and fourth layers. This finding is in general agreement with the earlier observations of Karpatkin, and we also find, as he did, that there is an inverse relationship between the centrifugal force at which PRP platelets are prepared and mean platelet volume. If the relation between platelet volume and age is valid, this technique may introduce substantial bias in the selection of platelets. While heavy platelets are larger, there is considerable heterogeneity of platelet volume among all density subgroups, an observation also made by Karpatkin. With increasing density, the distribution curve shifts, but the change in mean volume is quite small. The order of magnitude of volume change reported by us is similar to that reported in dogs by Minter and Ingram. Also of note is the wide range of platelet sizes among individuals and the overlap in size for various subpopulations. The difference between the two extreme populations in our study is much less than the 2 to 2.4-fold volume difference described by Karpatkin. He started with PRP which may have excluded some small but dense platelets, his methods of volume determination may have been less accurate, and the inert oils may have caused an alteration of platelet volume. These factors alone or in combination may account for the different results.

Conflicting hypotheses exist concerning the determinants of platelet heterogeneity. Earlier investigators using stimulated thrombopoiesis or suppressed thrombopoiesis have supported the idea that platelet volume heterogeneity is a reflection of platelet age. Minter and Ingram, using the acute hemorrhage model in dogs, could not confirm this. They showed instead that the large dense young platelets persisted for the entire platelet life span. Studies by Karpatkin, Amorosi, et al., and Charmatz and Karpatkin, using platelet cohort-specific radionuclides in man and rabbits during thrombopoietic equilibrium, have provided evidence that young platelets are denser and larger and progress to less dense smaller platelets. George and Sears, using a platelet membrane label, have shown progressive in vivo membrane loss with age, which tends to support the observed reduction of mean volume with increased age.

Other workers have emphasized the importance of events during thrombocytopoiesis as being the major determinant of platelet volume heterogeneity, as opposed to aging events in the peripheral circulation. von Behrens has shown that the large platelets seen in patients with hereditary Mediterranean macrothrombocytopenia are not necessarily young platelets.

We agree with Paulus that platelet size of a total population is heterogeneous, and we also find that the presumed heavy young platelets exhibit a heterogeneous size distribution. Thrombopoiesis does appear to be a major determinant of platelet volume, but for each individual there is a progressive reduction of platelet volume with decreasing density. Large platelets may not be synonymous with young platelets, but there is much evidence to suggest a cor-
relation between platelet age and volume, and the large platelets of Mediterranean macrothrombocytopenia may also undergo volume loss with aging. Changes in thrombopoiesis due to disturbances of thrombopoietic equilibrium may alter the magnitude and dispersion of platelet size, but these platelets still may be subjected to modifying factors in the peripheral circulation. Further evidence in favor of platelet modification with aging in the peripheral circulation is suggested by ultrastructural observations. The number of granules per platelet varies significantly with position in the gradient. If the relationship between cell age and density is valid, then this could be interpreted as an in vivo aging effect. The constant distribution of mitochondria per platelet, regardless of platelet density, suggests that variation of granule content is not merely the result of random organelle distribution at the time of platelet release. The reduction of platelet volume and total granule content with age may indicate that platelets can undergo nonfatal hemostatic interactions during senescence, as suggested, and raises the possibility that these interactions could be accelerated without necessarily curtailing net platelet survival.

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HUMAN PLATELET SUBPOPULATIONS


Heterogeneity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume, and ultrastructure

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